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A novel epiphytic cyanobacterium associated with reservoirs affected by avian vacuolar myelinopathy

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Abstract

Avian vacuolar myelinopathy (AVM) is a newly discovered bird disease, which is killing bald eagles (*Haliaeetus leucocephalus*) and waterfowl in the southeastern United States. Surveys were conducted to investigate exotic macrophytes (e.g. *Hydrilla verticillata*) as a substrate for attachment by toxic cyanobacteria that may be associated with the incidence of AVM. While the specific cause of the disease has not been confirmed, one hypothesis is that birds ingest a neurotoxin produced by cyanobacteria epiphytic on macrophytes. A strong relationship was found between the field abundance of a specific undescribed epiphytic cyanobacterium and the incidence of AVM. The undescribed species is a filamentous, heterocystous, true branching cyanobacterium. Morphological characteristics place the cyanobacterium in section V, order Stigonematales. The 16S rRNA sequence identity was determined from environmental isolates of this unknown Stigonematalan species using DGGE (denaturing gradient gel electrophoresis). The 16S rRNA sequence data were aligned with additional cyanobacteria sequences to determine designations for probe development, to lay groundwork for its formal description and to advance understanding of the species' phylogeny. Real-time PCR assays were developed for rapid, specific detection of the Stigonematales species from environmental samples. The genetic probe produced by this study will help test the hypothesized link between these cyanobacteria and AVM, and therefore help guide decisions on managing hydrilla and other invasive macrophytes in AVM-affected waters.

Keywords: Avian vacuolar myelinopathy (AVM); Cyanobacteria; *Fulica americana*; *Haliaeetus leucocephalus*; Hydrilla; Real-time PCR; Stigonematales; 16S

1. Introduction

Avian vacuolar myelinopathy (AVM) is a fatal disease of the nervous system that was first observed in bald eagles (*Haliaeetus leucocephalus*) in 1994 (Thomas et al., 1998). AVM has caused mortality in unknown numbers of bald eagles, American coots (*Fulica americana*), Canada geese (*Branta canadensis*), great horned owls (*Buba virginianas*), mallards (*Anas platyrhynchos*) and other species of birds found throughout the southeastern United States (Thomas et al., 1998). The peak month for AVM outbreaks is November; however, AVM has been diagnosed from October until April (Rocke et al., 2002).

Histologically, AVM has been characterized by spongy degeneration throughout the white matter of the central nervous system (Thomas et al., 1998). Vacuoles have been found throughout the white matter of the brain and were particularly severe in the optic lobes

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(Thomas et al., 1998). Tissues of infected birds have been analyzed for a variety of disease-causing agents including bacteria, viruses and parasites, as well as a wide range of toxins including organic metals, pharmaceuticals and plant toxins (Thomas et al., 1998). Lipophilic organic compounds from sediments and coot tissues in both AVM-affected and unaffected reservoirs have also been tested and no correlation was found (Dodder et al., 2003). None of the tests conducted has identified the causative agent of AVM-diseased birds. A point source exposure to a neurotoxin is regarded as the most likely cause of mortality (Rocke et al., 2002).

Although the specific cause of the disease has not been confirmed, studies have shown that waterfowl contract AVM via ingestion of submerged aquatic vegetation (*Hydrilla verticillata*) and associated biota (Birrenkott et al., 2004). Birds of prey contract the disease via ingestion of AVM-positive waterfowl tissue (Fischer et al., 2003). A strong association has been observed between the occurrence of AVM, *H. verticillata* (hydrilla) and certain potentially toxic, epiphytic cyanobacteria on hydrilla (Wilde et al., 2005). From 2001 to the present, surveys of freshwater reservoirs, lakes and ponds throughout the southeastern U.S. revealed that a species of potentially toxic cyanobacteria was present on the surface of hydrilla at every site where AVM had been diagnosed, but was rare or not found in areas where AVM was not observed (Wilde et al., 2005). The extent of plant surface covered by this cyanobacteria species (up to 95% of the leaf and stem surface of hydrilla) is correlated with prevalence of AVM disease (Wilde et al., 2005). This strong association between the occurrence of AVM, hydrilla and certain potentially toxic epiphytic cyanobacteria on hydrilla, led to the hypothesis that these epiphytes are the source of the neurotoxin causing AVM (Wilde et al., 2005).

Based on morphology, Wilde et al. (2005) described the epiphytic cyanobacteria suspected as the source of AVM neurotoxin as a novel Stigonematales species. The purpose of this study was to obtain a genetic sequence in order to develop a species-specific real-time PCR assay. Density gradient gel electrophoresis (DGGE) has been used to identify target sequence data from material collected in the field. Using methods described by Ferris et al. (1996), we have been able to isolate target 16S rRNA sequence data from DeGray Lake, AR and Lake Thurmond, SC from hydrilla covered with the novel Stigonematales cyanobacterium. The 16S rRNA sequence data were aligned with additional cyanobacteria sequences to determine designations for probe development, to advance understanding of the species' phylogeny, and to lay groundwork for its formal description.

2. Materials and methods

2.1. Culture maintenance

Targeted Stigonematales colonies were scraped off hydrilla leaves and streaked on nutrient agar plates containing BG-11 medium (ATCC medium 616), BG-11_0 medium (ATCC medium 819), BG-11_0 with added 10% hydrilla extract, or Chu's medium (ATCC medium 341). Plates were stored at 25 °C with light intensity of 75–85 $\mu E\ m^{-2}\ s^{-1}$ on a 12 h:12 h, light:dark cycle. Once colonies were established on agar, they were transferred onto fresh agar plates until a mono-specific culture of the targeted cyanobacterium was obtained. The targeted Stigonematales species was then transferred to liquid media. The liquid medium was BG-11_0 medium modified with 10% hydrilla extract. Hydrilla extract was made by wrapping hydrilla leaves in cheesecloth and boiling the leaves for 1 h in distilled water. The extract was then filtered and autoclaved. The liquid cultures were transferred to a larger incubator and stored at 27 °C with a light intensity of 60–80 $\mu E\ m^{-2}\ s^{-1}$ on a 16 h:8 h, light:dark cycle. Gradually, the culture was transferred to BG-11_0 media minus the hydrilla extract in order to establish a standardized culture medium. Culture isolates were obtained from hydrilla samples collected from DeGray Lake, Arkansas and Parksville Cove at Lake Thurmond, South Carolina. AVM was first documented in DeGray Lake. Parksville Cove was chosen because the hydrilla from AVM-positive mallard feeding trials (Birrenkott et al., 2004) was collected from this location.

2.2. Field collection

Samples of aquatic plants with epiphytic cyanobacteria were collected from AVM-positive locations (Table 1). Ziploc™ gallon (3.79 l) bags full of aquatic vegetation were taken from throughout the water column using a rake. From each subsample, 10 leaves covered with a heavy epiphytic growth were selected by eye. The underside of each leaf was scraped clean using a sterile razor blade and the scrapings were placed in a sterile 1.5 ml microcentrifuge tube containing 1 ml sterile CTAB (cetyltrimethylammonium bromide) buffer. Duplicate samples were processed for each site. The microcentrifuge tubes were stored at room temperature prior to DNA extraction.

Table 1
Field collection of hydrilla and associated cyanobacteria, and a *Hapalosiphon fontinalis* culture from the American type culture collection (Hap ATCC)

Lane	Sample name	Sample location	Date collected
1	Hap ATCC	ATCC culture isolate 39694	6 October 2003 (purchased)
2	DeRoche Sonicated A	DeGray reservoir, AR	6 October 2003
3	DeRoche Sonicated B	DeGray reservoir, AR	6 October 2003
4	DeRoche 1A	DeGray reservoir, AR	6 October 2003
5	DeRoche 2A	DeGray reservoir, AR	6 October 2003
6	DeRoche 3A	DeGray reservoir, AR	6 October 2003
7	Parksville 1A	Lake Thurmond, SC	8 October 2003
8	Parksville 2A	Lake Thurmond, SC	8 October 2003
9	Parksville 3A	Lake Thurmond, SC	8 October 2003
10	Susie Ebert A	Lake Murray, SC	21 November 2002
11	Spence Houseside A	Lake Murray, SC	21 November 2002
12	Woodlake Marina	Woodlake, NC	18 November 2002
13	Woodlake 4	Woodlake, NC	18 November 2002
14	Thurmond CHR05 A	Lake Thurmond, SC	7 November 2002
15	Thurmond Clark's Hill A	Lake Thurmond, SC	7 November 2002
16	Thurmond Clark's Hill B	Lake Thurmond, SC	7 November 2002
17	Thurmond C	Lake Thurmond, SC	7 November 2002
18	Thurmond CHR05 B	Lake Thurmond, SC	7 November 2002

Samples were collected for DNA extraction and 16S rRNA analysis. Lane number correlates with the DGGE gel lane and label (Fig. 2).

2.3. DNA extractions of the targeted cyanobacterium

Environmental samples (Table 1) were extracted using a rapid CTAB buffer DNA isolation technique (Schaefer, 1997). Briefly, samples in CTAB buffer were transferred to sterile 15 ml polypropylene tubes and homogenized using a sterile wooden applicator stick. Samples were incubated for 2 h at 60–65 °C. Chloroform and isoamyl alcohol mixture (24:1) were added to each 15 ml tube with homogenized material. Tubes were vigorously shaken to mix. Samples were immediately centrifuged at 4500 rpm for 30 min. A 850 μl of the top aqueous layer was removed from each 15 ml tube and placed in a sterile 1.5 ml centrifuge tube. Six hundred microliters of 2-propanol was added to each 1.5 ml tube and the tube inverted 15 times to mix. Tubes were placed in a microcentrifuge and spun for 30 min at 14,000 rpm. The supernatant was poured off and the pellet air-dried for 1–2 h. The DNA pellets were resuspended with 20 μl of sterile water and allowed to sit overnight at 4 °C. Extracted DNA was stored at −20 °C.

2.4. PCR for DGGE analysis

Extracted DNA from environmental samples and culture isolates obtained from DeGray Lake and Parksville Cove were selected to sequence the 16S rRNA from the targeted cyanobacteria species. The sequencing primers were selected from a previous study for profiling microbial mat communities (Ferris et al., 1996). The forward primer (1055F) complements a region conserved among members of the domain bacteria (*Escherichia coli* positions 1055–1070; primer sequence 5′-ATGGCTG-TCGTCAGCT-3′. The reverse primer (1406R) is based on a universally conserved region and incorporates a 40-base GC clamp (*E. coli* positions 1393–1406; 5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCC-CCCG CCCCACGGGCGGTGTGTAC-3′); Ferris et al. (1996). PCR was used to amplify the 16S rRNA gene from environmental isolates and cyanobacteria cultures. The following reagents were added to each 25 μl reaction mixture: 0.2 μm primers; 1× PCR buffer (Promega, Madison, WI); 2.5 mM $MgCl_2$, 0.2 mM dNTP mixture (Stratagene, La Jolla, CA); 10 μm bovine serum albumin; 0.2 U μl^{-1} *Taq* polymerase (Promega, Madison, WI). The reaction conditions for amplification were 94 °C for 5 min, followed by 20 touchdown cycles of 30 s at 94 °C, 30 s at 53 °C, decreasing by 0.5 °C each cycle, and 2 min at 72 °C. The reaction continued for an additional 20 cycles of 30 s at 94 °C, 30 s at 43 °C, and 2 min at 72 °C, followed by a 6 min extension at 72 °C and a final holding cycle at 4 °C. PCR products were verified by 1% agarose gel electrophoresis and purified with QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA).

2.5. DGGE

PCR products were analyzed by DGGE using a Dcode Universal Mutation Detection System (Bio-Rad

Laboratories, Richmond, VA), following Muyzer et al. (1993). Briefly, samples were run on an 8% polyacrylamide gel in 1× TAE containing a 40–60% denaturing gradient (100% is 7 M urea and 40% formamide). Electrophoresis was carried out for 10 h at 100 V at 60 °C. The gels were stained for 1 h with SYBR Green I (Molecular Probes, Eugene, OR) and analyzed using a Typhoon 9410 imaging system (Amersham Biosciences, Buckinghamshire, UK).

2.6. Band isolation

Bands observed in the DGGE community profile were excised with a sterile pipette tip and the DNA was eluted in 30:1 sterile deionized water. The eluted DNA was reamplified as previously described above except that the reverse primer, 1406R, lacked the 40 bp GC clamp. Amplified DNA was purified using QIAquick PCR purification columns. The PCR product was verified by 1% agarose gel electrophoresis and viewed with the Bio-Rad Model 1000 VersaDoc gel imaging system (Bio-Rad Laboratories Inc., Hercules, CA).

2.7. Sequencing

DNA was sent to Nevada Genomics Center (NGC) at the University of Nevada, Reno for quantification and sequencing.

2.8. PCR primer development for sequencing

The initial DGGE sequence results from NGC were downloaded and aligned. The sequence alignments were created by eye using BioEdit Sequence Alignment Editor (Hall, 1999). The chromatograms were used to resolve any ambiguities in the sequence data. The initial sequence was submitted to the advanced BLAST search program National Center for Biotechnology Information (NCBI) for determination of most similar sequences (Altschul et al., 1990). The initial sequence was aligned with 48 other cyanobacteria species for selection of potential primers for obtaining the remainder of the 16S rRNA sequence (Table 2). Primers were developed based on nucleotide differences between the initial sequence of the suspect cyanobacterium and the 48 other aligned cyanobacteria species. Each candidate primer was analyzed in Gene Runner ver. 3.05 (Hastings Software Inc., Hudson, NY). Primers were approximately 20 bp in length and are listed in Table 3. PCR was used to amplify the 16S rRNA gene from environmental isolates and the Hap ATCC culture. The same reagents and method was used as previously described.

2.9. PCR purification for sequencing

Two methods were used for purifying PCR products for sequencing. If the target band (desired PCR product based on relative positioning to the molecular marker, HiLo DNA Marker, Minnesota Molecular) was the only band in the respective lane, then the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) was used to purify the PCR products for sequencing. If bands were present in addition to the target band, then the target band was excised using a sterile razor blade, placed in a sterile microcentrifuge tube, and DNA was extracted using the NucleoSpin Extraction Kit (BD Bioscience, San Jose, CA). Purified PCR product was then sent to Nevada Genomics for sequencing. The previously described sequencing procedure was followed.

2.10. Sequencing alignment

Sequence data from NGC were downloaded and aligned. A multiple alignment of sequences was performed using the CLUSTAL X ver. 1.83 program (Thompson et al., 1997). Sequence alignments were confirmed using BioEdit Sequence Alignment Editor 5.0.9 (Hall, 1999). Chromatograms were used to resolve any ambiguities in the sequence data. A final consensus sequence was created in BioEdit and submitted to GenBank.

The final consensus sequence of the cyanobacterium was submitted to the advanced BLAST search program NCBI for determination of most similar sequences (Altschul et al., 1990). A multiple alignment of the most similar species was performed using the CLUSTAL X ver. 1.83 program (Thompson et al., 1997) using the IUB weight matrix. The resulting alignment was then confirmed and aligned by eye using BioEdit Sequence Alignment Editor 5.0.9 (Hall, 1999).

2.11. Primer and probe selection for real-time PCR

The consensus sequence was visually compared to the rRNA sequence of the other 48 cyanobacteria species (Table 2) for selection of potential primers for real-time PCR. Primers were chosen based on nucleotide differences between the consensus sequence and the other sequences at the variable regions. Each candidate primer was analyzed in Gene Runner ver. 3.05 (Hastings Software Inc., Hudson, NY). The final primer and probes were submitted to and compared in

Table 2
16S rRNA sequence data obtained from GenBank

GenBank accession nos.	Organism/16S rRNA gene	GenBank reference
AP006573, BA000045	*Gloeobacter violaceus* PCC 7421	Nakamura et al. (2003)
AJ293110	*Anabaena* cf. *cylindrica* 133	Gugger et al. (2002)
AB074502	*Anabaena variabilis*	Seo and Yokota (2001)
X59559	*Anabaena* sp.	Ligon et al. (1991)
AB119259	*Aphanothece sacrum*	Horiguchi et al. (2003)
AB074504	*Calothrix brevissima*	Seo and Yokota (2001)
AF132777	*Chlorogloeopsis* sp. PCC 6718	Turner et al. (1999)
X68780	*Chlorogloeopsis* sp.	Van Der Auwera (1992)
AY038036	*Cyanospira rippkae*	Iteman et al. (2002)
AJ000708	*Cyanothece* sp. PCC 7418	Nubel et al. (1998)
AJ133163	*Cylindrospermum stagnale* PCC 7417	Lyra et al. (2001)
AY218831	Cylindrospermum sp. CENA33	Fiorel et al. (2003)
AJ344560	*Fischerella* sp. BB98.1	Fewer et al. (2002)
AF132788	*Fischerella muscicola*	Turner et al. (1999)
AJ544076	*Fischerella* sp.	Gugger and Hoffmann (2004)
AB067578	*Gloeocapsa* sp. KO20B5	Ohki et al. (2001)
AY034793	*Hapalosiphon welwitschii*	Hoffmann and Troelstrup (2001)
AB093484	*Hapalosiphon delicatulus* IAM M-266	Seo and Yokota (2002)
AJ544078	*Hapalosiphon* sp. 804-1	Gugger and Hoffmann (2003)
X84809	*Leptolyngbya* sp.	Nelissen et al. (1996)
AJ133174	*Microcystis wesenbergii* NIES-104	Lyra et al. (2001)
AB035550	*Microcystis ichthyoblabe*	Otsuka et al. (1999)
AY038034	*Nodularia spumigena* PCC 9350	Iteman et al. (2002)
AB093490	*Nostoc entophytum* IAM M-267	Seo and Yokota (2002)
AP003598, BA000019	*Nostoc* sp. PCC 7120	Kaneko et al. (2001)
AB074503	*Nostoc linckia*	Seo and Yokota (2001)
AJ544081	*Nostochopsis* sp. 89-45	Gugger and Hoffmann (2004)
AJ544080	*Nostochopsis lobatus* 92.1	Gugger and Hoffmann (2004)
AJ133165	*Oscillatoria* sp. 28	Lyra et al. (2001)
AB003169	*Phormidium* sp.	Ishida et al. (1997)
AB003167	*Phormidium ambiguum*	Ishida et al. (1997)
AB074507	*Planktothrix agardhii*	Seo and Yokota (2001)
X78681	*Pleurocapsa* sp. PCC 7516	Nelissen et al. (1995)
AJ007907	*Prochlorothrix hollandica*	Zwart et al. (1998)
AJ544082	*Stigonema ocellatum* SAG 48.90	Gugger and Hoffmann (2004)
AJ544084	*Symphyonema* sp. 1517	Gugger and Hoffmann (2004)
AJ544083	*Symphyonema* sp. 1269-1	Gugger and Hoffmann (2004)
AJ544085	*Symphyonemopsis* sp. VAPOR1	Gugger and Hoffmann (2004)
AJ000716	*Synechococcus* sp. PCC 7002	Nubel et al. (1998)
AB093486	*Tolypothrix* sp. IAM M-259	Seo and Yokota (2001)
AF013028	*Trichodesmium contortum*	Janson et al. (1999)
AF516748	*Umezakia natans*	Neilan et al. (2003)
AJ544222	*Westiellopsis* sp. 89-785/4	Gugger and Hoffmann (2004)
AJ544090	*Westiellopsis* sp. 985-1	Gugger and Hoffmann (2004)
AB074510	*Xenococcus* sp. PCC 7307	Seo and Yokota (2001)

Sequences were chosen based on morphological characteristics and BLAST results from the comparison of the unknown cyanobacterium (GenBank accession no. AY785313). Sequence information was used for PCR-primer development.

the NCBI database in order to eliminate matches with other known species. Any primer sequence that found a matching sequence in the NCBI database was eliminated as a candidate primer. Final forward and reverse primers and a fluorescently labeled internal probe were chosen and are listed in Table 3. The internal probe was labeled on the 5′ end with 5-carboxyfluorescein and the 3′ end with 5-carboxytetramethylrhodamine (QIAGEN Operon, Alameda, CA).

2.12. Real-time PCR analysis

From each environmental location tested, 0.5 g of hydrilla leaves were weighed and placed in a 1.5 ml

Table 3
Developed primers

Primer name	5′–3′ sequence	Reference
8 (forward)	5′-AGAGTTTGATCCTGGCTCAG-3′	Wilmotte et al. (1993)
375 (forward)	5′-CTCCTACGGGAGGCAGCAG-3′	Wilmotte et al. (1993)
1100 (reverse)	5′-AGGGTTGCGCTCGTTG-3′	Wilmotte et al. (1993)
16 Reverse	5′-AAGGAGGTGATCCAGCCGCA-3′	Wilmotte et al. (1993)
Stig 226 Reverse	5′-AGTCTGGACCGTGTCTCAGT-3′	This study
Stig 349 Reverse	5′-TACCTTCTCTATTATTCCCTGA-3′	This study
Stig 324 Reverse	5′-CTGCTGCCTCCCGTA-3′	This study
Stig 932 Forward	5′-CGCGAATCTTGATGAAAGT-3′	This study
Stig 1245 Forward	5′-AAATCTCGTAAACCGTTGC-3′	This study
Stig 1260 Reverse	5′-TTGCTCAGTTCAGACGAAG-3′	This study
Stig 1263 Reverse	5′-GCAACGGTTTACGACATTT-3′	This study
Stig 1279 Reverse	5′-CCTTCGATCTGAACTGAGCAA-3′	This study
Stig 1069 Forward	5′-AGGGTGGGCACTCTAAAGA-3′	This study for RT-PCR
Stig 1248 Reverse	5′-CAGCCTTCGATCTGAATTG-3′	This study for RT-PCR
Stig 1209 Probe	5′[6∼FAM]CAAATCTCGTAAACCGTTGCTAATT[TAMRA∼6∼FAM] 3′	This study for RT-PCR

Primers were selected or designed to target the 16S rRNA gene of the cyanobacterium. Primers were used for sequencing and real-time PCR probe development.

tube containing 1 ml of CTAB buffer. DNA for real-time PCR was extracted using a rapid CTAB buffer DNA isolation technique (Schaefer, 1997) as previously described. All real-time PCR assays were conducted using the SmartCyler® System (Cepheid, Sunnyvale, CA). Reaction protocols for each assay consisted of three stages, two denaturation stages at 96 °C for 75 s, 50 cycles at 96 °C for 5 s, and an annealing stage at temperatures ranging from 58 to 63 °C for 40 s. Reaction mixtures from each assay consisted of the developed two flanking primers and the internal fluorescent-labeled probe (Table 3). The following reagents were added to each 25 μl reaction mixture: 0.2 μm primers and internal probe; 1× PCR buffer (Promega, Madison, WI), 2.5 mM $MgCl_2$, 0.2 mM dNTP mixture (Stratagene, La Jolla, CA), 10 μM bovine serum albumin, and 0.2 U μl^{-1} *Taq* polymerase (Promega, Madison, WI). The species-specific real-time PCR assay was optimized using culture and environmental samples containing the targeted cyanobacteria species as positive controls (Table 4) and extracted DNA from 13 freshwater cyanobacteria species as negative controls (Table 5).

3. Results

3.1. Culturing and microscopy

Culture isolates from DeGray Lake and Parksville Cove had no discernable morphological differences. Microscopic examination of the targeted cyanobacterium growing epiphytically on hydrilla leaves and targeted cyanobacterial culture isolate (Fig. 1) revealed diagnostic morphological characteristics. The targeted cyanobacterium is a sheathed, T type true branching, colony-former.

The cyanobacterium initially grew best in liquid BG-11_0 medium. The cyanobacterium grew slowly or was

Table 4
DNA samples used for real-time PCR optimization

Reaction number	Sample name	Source	Presence of Stigonematales species
1	Control	No DNA used	Negative
2	Culture A (SCAEL-Stig-2004)	Cultured environmental isolate	Positive
3	Culture B (SCAEL-Stig-2004)	Cultured environmental isolate	Positive
4	Parksville	Lake Thurmond, SC	Positive
5	DeRoche	Lake DeGray, AR	Positive
6	Thurmond	Lake Thurmond, SC	Positive
7	Susie Egbert	Lake Murray, SC	Positive
8	Spence Houseside	Lake Murray, SC	Negative
9	Woodlake	Woodlake, NC	Positive

The presence of the Stigonematales species was confirmed via microscopy prior to real-time PCR analysis.

Table 5
Negative controls used in testing the accuracy of the real-time PCR assay

	Cyanobacteria	ID, culture, source
1	*Anabaena variabilis*	Bluffton, IN 2001
2	*Lyngbya martensiana*	Cyano #54 Bluffton, IN 2001
3	*Phormidium ambiguum*	Cyano #10B 65-13-98-512-3-AR6 IN
4	*Oscillatoria amoena*	Cyano #16 6-5-98-5-28-13 IN
5	*Oscillatoria deflexoides*	Cyano #8 IN
6	*Oscillatoria jasorvensis*	Cyano #7 IN
7	*Oscillatoria limosa*	Cyano #4 IN
8	*Oscillatoria pseudogeminata*	Cyano #60 Bluffton, IN 2001
9	*Lyngbya martensiana*	Cyano #50 Bluffton, IN 2001
10	*Pseudanabaena limnetica*	Eagle Creek Reservoir Indianapolis, IN 2001
11	*Pseudanabaena* sp.	Cyano-Florida, Andrew Chapman, Green Water Laboratory
12	*Microcystis aeruginosa*	UTEX LB 2385
13	*Hapalosiphon fontinalis*	ATCC 39694

The cyanobacteria isolates #1–10 were isolated by and obtained from Dr. Carole Lembi (Purdue University). All samples tested negative. ATCC, American type culture collection; UTEX, University of Texas Culture Collection of Algae.

outcompeted on the Chu's medium and BG-11 medium. Once the monoculture was established, the cyanobacterium grew best in BG-11 medium. Growth patterns differed slightly in media versus hydrilla as a substrate. On solid media ($BG\text{-}11_0$), the alga formed small dense greenish colonies with filaments branching in all directions, but were mainly erect. The colony formation was three-dimensional rather than starting out in a single plane. Branching occurred in all planes from the basal filaments. Mature colonies were large enough to see with the unaided eye. Colonies typically developed long rounded to spiraled formations. Occasionally colonies grew in a thinner, one-dimensional mass.

Morphological characteristics place the targeted cyanobacterium in the order Stigonematales based on Rippka et al. (1979) classification standards. Characteristics include true-branching, heterocysts, hormogonia, and filaments enclosed by a sheath. Genus and species identification could not be determined based on morphology. Culture and environmental samples were sent to Dr. Jiri Komárek (University of South Bohemia, Czech Republic), who concluded (personal communication) that the cyanobacterium is morphologically most similar to three Stigonematales genera*: Hapalosiphon*, *Fischerella*, or *Thalpophila*.

Morphological and genetic comparisons with *Hapalosiphon fontinalis* ATCC 39694 were made in order to better classify the unknown Stigonematales species. Morphological comparisons of the targeted cyanobacterium with *H. fontinalis* revealed distinct differences. *H. fontinalis* had longer, thinner trichomes that spiraled through the media. *H. fontinalis* also branched throughout the medium, rather than forming dense mats. Also, individual cells of *H. fontinalis* were rounder and smaller (2 μm × 5 μm on average) than the environmental isolate (5 μm × 10 μm on average).

3.2. 16S rRNA amplification

Amplification of the 16S rRNA gene of the Stigonematales species was achieved using combinations of universal primers and species-specific primers developed here (Table 3). The initial targeted sequence was obtained from the DGGE gel (Fig. 2). Both DeGray Lake and Parksville Cove environmental isolates (lanes 2–7 of Fig. 2) were sequenced and aligned using primers listed in Table 3. These samples were selected for sequencing based on the age of the sample and clarity of the specific banding patterns. The sequenced fragments were compared to sequences listed in GenBank in order to confirm that the fragments were most genetically similar to cyanobacteria.

Initial sequences derived from DGGE bands were used to develop species-specific primers that were used to sequence the remaining portion of the 16S gene. Developed primers were successful in amplifying remaining sections of the 16S gene. New PCR products were run on agarose gels in order to confirm that the developed primer sets worked prior to sequence submission. Sequence differences were not found between DeGray Lake and Lake Thurmond isolates, therefore a consensus sequence was created.

The culture isolate also was sequenced using the same developed primers. Agarose gels were run prior to culture sequence submission in order to confirm that the primer sets amplified DNA. Sequence data from environmental and culture isolates were aligned and differences were not found, evidence that they are the

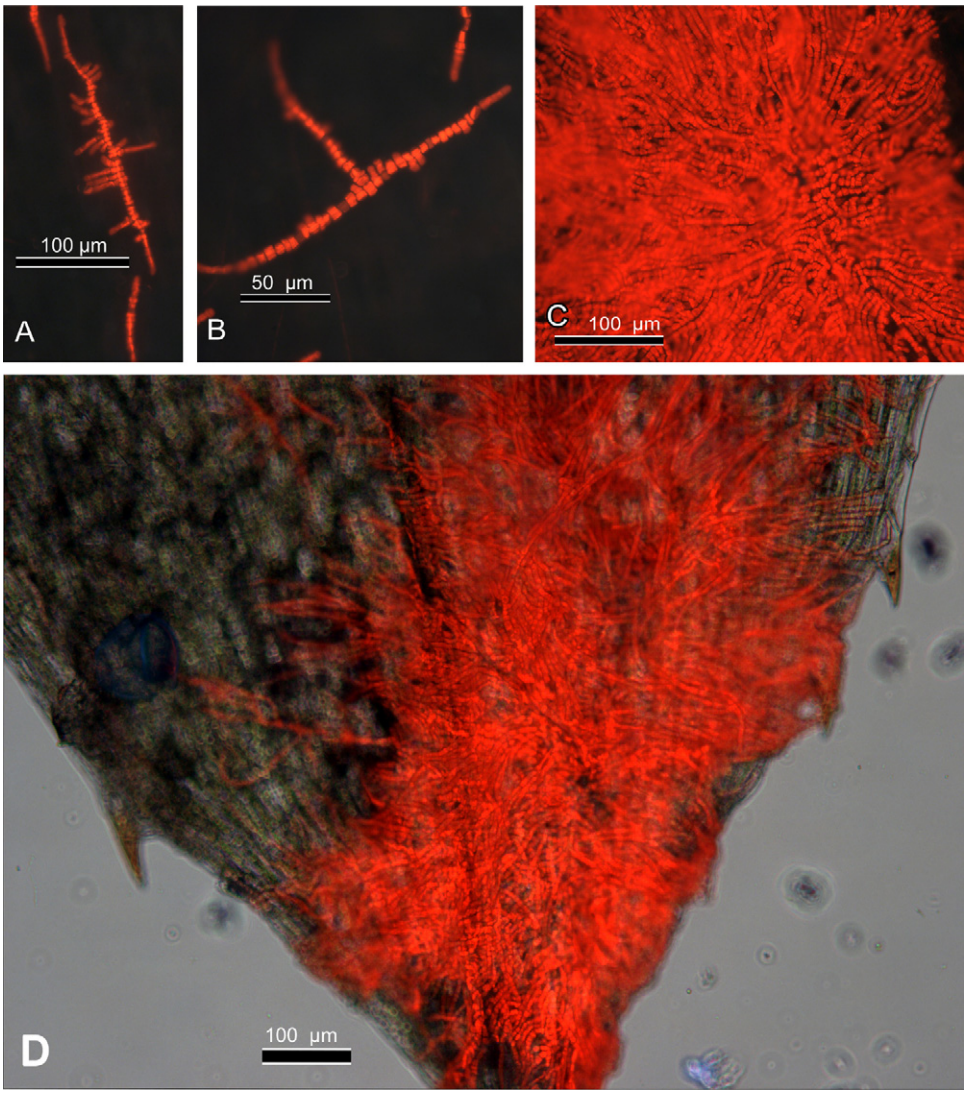

Fig. 1. Stigonematales species growing epiphytically on the underside of hydrilla leaves. Images taken using epifluorescence with a rhodamine red filter. (A and B): note true branching from both sides of the basal filament. (C): Large true branching filamentous colony. The majority of filaments are in the same plane with the leaf surface. (D): Hydrilla leaf with attached cyanobacterial colonies. Brightfield microscopy with epifluorescence and a rhodamine red filter.

same species. Using the sequencing primes described, 1480 bp were sequenced for the Stigonematales species. The consensus sequence was generated and initially evaluated using BLAST program (Altschul et al., 1990) against published sequences in GenBank. No matches resulted. The consensus 16S rRNA sequence was deposited in GenBank with the accession no. AY785313 (Habrun et al., 2004).

Lane one of the DGGE gel (Fig. 2) contained DNA from a culture of *H. fontinalis,* ATCC 39694. Because the banding pattern is unique for *H. fontinalis* in lane 1, this indicates that *H. fontinalis* is genetically different from the AVM-targeted species (lanes 2–18).

3.3. Similarity of the Stigonematales species 16S rRNA sequence to other GenBank sequences

The full length 16S rRNA sequence (1480 bp) for the Stigonematales species was compared to other rRNA sequences in GenBank. A BLAST search of GenBank found 10 organisms with a similarity ranking of 0.94 or higher (Table 6). A second similarity search was conducted in GenBank with a smaller portion of the Stigonematales species (1300 bp). The smaller subset yielded similar results with 11 species having similarity of 0.94 or higher (Table 7). The most similar sequence in both lists is an

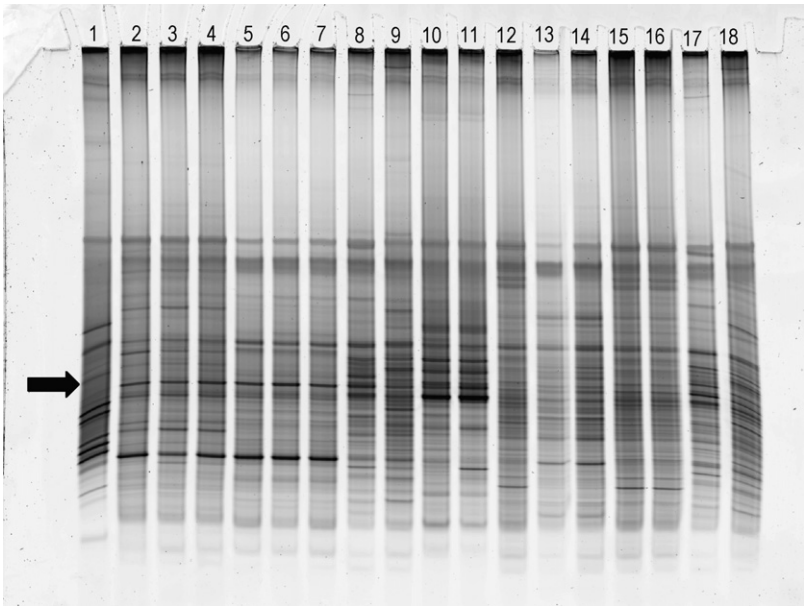

Fig. 2. Denaturing gradient gel electrophoresis products for the 16S rRNA amplification of cultures and environmental samples. Lane identification corresponds to Table 1. The arrow indicates the Stigonematales species band that was sequenced.

uncultured bacterium (GenBank accession no. AB179514, Yoshida et al., 2004, unpub.) that was isolated from a microbial environment associated with iron reduction/oxidation processes in siliceous sedimentary rock. No morphological or classification information was given for the bacterium. The second most genetically similar species is *Stigonema ocellatum* (GenBank accession no. AJ544082, Gugger and Hoffmann, 2004), which was obtained from a sphagnum bog in Germany. *S. ocellatum* is in the order Stigonematales. The remaining most genetically similar species are all classified in the order Nostocales, the false branching order.

3.4. Real-time PCR probe development and optimization

The unknown Stigonematales species sequence was aligned with 48 cyanobacteria species in Table 2 according to nucleotide sequences in conserved regions. Gaps were inserted into regions to improve alignment of the cyanobacteria. The majority of nucleotide variability was seen in the 1050–1300 base region, and therefore this was the region from which the Stigonematales species-specific primers were selected (Table 3). When analyzed using BLAST algorithm, the primers did not generate a match with any of the sequences in the GenBank database. Once the species-specific primers had been

Table 6
Similarity ranking for the Stigonematales species sequence

GenBank accession nos.	Organism/16S rRNA gene	Similarity ranking	GenBank reference
AB179514	Uncultured bacterium	0.979	Yoshida et al. (2004)
AJ544082	*Stigonema ocellatum* SAG 48.90	0.949	Gugger and Hoffmann (2004)
AJ133163	*Cylindrospermum* sp. PCC 7417	0.945	Lyra et al. (2001)
AF132789	*Cylindrospermum stagnale* PCC 7417	0.944	Turner et al. (1999)
AB093486	*Tolypothrix* sp. IAM M-259	0.943	Seo and Yokota (2001)
X59559	*Anabaena* sp.	0.942	Sako et al. (1991)
AP003598	*Nostoc* sp. PCC 7120	0.942	Kaneko et al. (2001)
AB074502	*Anabaena variabilis*	0.94	Seo and Yokota (2001)
AJ293110	*Anabaena* cf. *cylindrica* 133	0.939	Gugger et al. (2000)

The 10 most genetically similar species to the entire 16S rRNA sequence (1480 bp) of the Stigonematales species (GenBank accession no. AY785313) generated by a BLAST search.

Table 7
Similarity ranking for the Stigonematales species sequence

GenBank accession nos.	Organism/16S rRNA gene	Similarity ranking	GenBank reference
AB179514	Uncultured bacterium	0.98	Yoshida et al. (2004)
AJ544082	*S. ocellatum* SAG 48.90	0.947	Gugger and Hoffmann (2004)
AF132789	*Cylindrospermum stagnale* PCC 7417	0.946	Turner et al. (1999)
AB093486	*Tolypothrix* sp. IAM M-259	0.946	Seo and Yokota (2001)
AJ133163	*Cylindrospermum* sp. PCC 7417	0.945	Lyra et al. (2001)
X59559	*Anabaena* sp.	0.943	Sako et al. (1991)
AP003598	*Nostoc* sp. PCC 7120	0.943	Kaneko et al. (2001)
AB074502	*Anabaena variabilis*	0.943	Seo and Yokota (2001)
AJ293110	*Anabaena cf. cylindrica* 133	0.94	Gugger et al. (2000)
AF247593	*Anabaena variabilis* NIES23	0.94	Beltran and Neilan (2000)

The 11 most genetically similar species to the partial 16S rRNA sequence (1300 bp) of the Stigonematales species (GenBank accession no. AY785313) generated by a BLAST search.

designed, it was important to test the forward and reverse primers and the internal probe. The probe primer set was optimized and found to successfully amplify the proper gene sequence of the targeted cyanobacterium. The final annealing temperature was optimal at 58 °C, and replicated for 45 cycles rather than 50 cycles. Optimization of the primer set was tested on 16 DNA samples listed in Table 4. Environmental samples (PCR reaction #4–9, Table 4) were examined via microscopy prior to real-time PCR analysis to determine presence or absence of the unidentified Stigonematales species. Two culture isolates and five positive environmental sites amplified as anticipated. The remaining nine environmental and culture isolates that did not obtain the target Stigonematales species were negative. Once optimization was complete, the primer/probe set was tested with 12 freshwater cyanobacteria species as controls (Table 5), which all remained negative.

4. Discussion

Although an association of AVM with hydrilla and epiphytic cyanobacteria had been established (Birrenkott et al., 2004; Wilde et al., 2005), the cyanobacterium proposed as the source of AVM neurotoxin had been only cursorily characterized prior to this study. Identification of the cyanobacterium and the ability to test its hypothesized role as the causative agent of AVM had been hampered by the lack of established pure cultures. A monoculture of the targeted Stigonematales species was established using BG-11_0 media. This medium does not contain nitrogen, and the ability of the Stigonematales species to out-compete co-occurring algae in BG-11_0, but not BG-11 medium, may relate to its nitrogen-fixing capability. Once the monoculture was established, the cyanobacterium was grown in BG-11 and BG-11_0. Growth was more successful in the media containing the nitrogen. Cells were a healthier green compared to the browner color in medium without nitrogen. Growth was not observed in Chu's medium, suggesting that substances present in BG-11, but not Chu's medium, were essential for growth of the Stigonematales species (e.g. $CaCl_2$ or the trace metal solution).

When combined, morphological and genetic analytical results indicated that the cyanobacterium associated with AVM is an undescribed species. Morphologically, the identity could only be narrowed down to three genera (*Hapalosiphon*, *Fischerella*, or *Thalpophila*). The 16S rRNA sequence did not match any of the GenBank deposits. Blast results from GenBank also indicate the species is in the order Stigonematales. This study yielded preliminary work for the formal description of this unidentified Stigonematales species.

The origin and history of the novel cyanobacterium remains unclear. The cyanobacterium may be a newly evolved species or newly introduced to the area via invasive hydrilla. Areas such as Asia, where hydrilla originates, should be examined for this novel Stigonematales species. If the recent (from 1994 to the present) occurrence of AVM is linked to the introduction or increased biomass of the Stigonematales epiphyte, then the future impact of AVM may increase as the cyanobacteria's host, hydrilla, continues to spread.

A genetic detection assay for the Stigonematales species was developed during this study. Real-time PCR results demonstrated that the assay was successful in detecting and amplifying culture isolates and environmental samples. The real-time PCR assay should allow accurate identification of the Stigonematales species in environmental and bird gastrointestinal samples, and enhance early detection of the Stigonematales species at potential disease sites.

This study yielded several contributions to the study of AVM. The identification of the cyanobacterium and

gene-probe development will improve testing of the hypothesized link between AVM and the Stigonematales species. The development of the culture will enable future feeding trials based on the Stigonematales species as sole disease source (i.e. without the hydrilla host), and aid characterization of the putative toxin. In general, these advancements will help determine whether the Stigonematales species is the cause of AVM and guide management decisions on mitigating this disease.

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